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# Improvements to the Forensic Analysis of Mitochondrial DNA Typing

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# Improvements to the Forensic Analysis of Mitochondrial DNA Typing

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Honors Senior Thesis

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**Abstract:** Sequence analysis of human mitochondrial DNA (mtDNA) is an effective and reliable tool for the genetic characterization of forensic samples. The nature of the mitochondrial genome (mtgenome), its high copy number and small size (~17kb) makes it more resistant to degradation and more stable than nuclear DNA. For this reason mitochondrial DNA is often the only feasible option for the forensic analysis of environmentally compromised samples. Currently the forensic analysis of the mtgenome is restricted to the hypervariable regions, also known as the Displacement loop (d-loop). Previous studies, confirmed in the Strausbaugh lab, have demonstrated an increased variability in the hypervariable regions between and amongst various populations. Within the D-Loop is the HV1, which is used forensically to include or exclude suspects. Current data suggest that analysis of the HV1 region has a low power of discrimination. However, the data is largely supported by samples of European ancestry and does not consider the variation observed in other populations. My research addresses this prediction through the evaluation of a forensically relevant population, self-identified U.S. African Americans. In several instances we were unable to amplify the region of interest due to problems with the current protocols and reagents. In order to answer the question of hypervariable region variation PCR reagents and appropriate amplification cycles had to be optimized. This research employs mtDNA hypervariable region amplification in conjunction with standard Sanger sequencing in order to evaluate the power of discrimination of mtDNA analysis. Results suggest that HV1 analysis is sufficient for many U.S. African American samples and that the power of discrimination of mtDNA analysis is based on the maternal ancestry of an individual

## **Introduction:**

### **Mitochondria**

Mitochondria are known as the organelles responsible for energy generation in cells. Enzymes within the outer and inner membranes of the mitochondria assist in converting materials into adenosine triphosphate (ATP) which fuels the metabolic activities of the cell (Davidson 2010). The mitochondrion is the location in which the final phases of aerobic respiration take place. The citric acid cycle occurs in the mitochondrial matrix while ATP synthase and enzymes of oxidative phosphorylation work across an electrochemical gradient of the tightly folded inner membrane (McBride, Neuspiel & Wasiak 2006). This network enables cells to use aerobic respiration to produce approximately 15 times more ATP than anaerobic respiration (Davidson 2010). Also in the mitochondrion, carbohydrates and long-chain fatty acids are broken down, adenosine diphosphate (ADP) and ATP are constantly converted into one another, steroids are synthesized, lipids are produced, the organelle's DNA is replicated, and mitochondrial proteins are translated (McBride, Neuspiel & Wasiak 2006). Research has indicated that mitochondria may be intimately involved in signaling cascades, cell cycle control, and dynamic moderation of respiratory capacity; this demonstrates that mitochondria function beyond their primary role of producing ATP (McBride, Neuspiel & Wasiak 2006). While most believe the mitochondria's role is limited to energy, scientific literature promotes a more dynamic function of the organelle.

Mitochondria are found in nucleated cells in most eukaryotes; this includes plants, fungi, animals, and multi-cellular protists (Davidson 2010). Human cells have a varied number of mitochondria, depending on the metabolic requirements of that particular cell (Davidson 2010). Some cells, like that of ascidian spermatozoa, yeast, and green algae, contain one mitochondrion; whereas, in humans, heart and muscle cells can contain thousands of mitochondria (Bereiter-Hahn & Vöth 1994). Muscle, for example, generally contains mitochondrial dense clusters near blood capillaries (Bereiter-Hahn & Vöth 1994).

These tissues that are high in “respiratory activity” have mitochondria with a greater amount of foldings which enhance surface area (Bereiter-Hahn & Vöth 1994).

### **Mitochondrial Morphology**

The mitochondrion’s complex compartmental structure is reflective of its dynamic function in energy production. It has two membranes that separate into four distinct compartments with each membrane-bound section being able to work cohesively to generate ATP (McBride, Neuspiel & Wasiak 2006). The two membranes divide the organelle into a narrow intermembrane space and a large internal matrix (Figure 1) (Davidson 2010). The outer membrane of the mitochondrion contains channel proteins and porins which selectively filter cellular components. The inner mitochondrial membrane consists of extensive foldings called cristae (Davidson 2010). The inward folds of the cristae increase the surface area available for enzymes involved in cellular respiration (Davidson 2010). The complexes of the electron transport chain and ATP synthase are found here as well as transport proteins that only allow the essential molecules into the matrix (McBride, Neuspiel & Wasiak 2006). It is these proteins that control the rate of metabolism in the cell (McBride, Neuspiel & Wasiak 2006).

Mitochondria can be found in a variety of different morphologies in mammals (Okamoto & Shaw 2005). They range in shape from long tubules connected to one another to small separate spheres (Okamoto & Shaw 2005). Mitochondria also range in size, between approximately 1 to 10 micrometers, but are known to fluctuate in both size and morphology regularly (Davidson 2010). It is understood that the organelles are attached to cellular microtubules which influence size, morphology, and locomotion in the cytoplasm (Davidson 2010). The cellular microtubules enhance mitochondria organization in formations that are beneficial for the cell based on its particular needs (Davidson 2010). Often, mitochondria can come into contact with one another while migrating along the microtubular network, causing the two ends to come together and fuse (Chan 2006). The mitochondrion’s “steady-state

morphology” is regulated by a collaboration of motility, fission, and fusion (McBride, Neuspiel & Wasiak 2006).

### **Fusion and Fission of the Mitochondria**

Mitochondria are semi-autonomous organelles, in that, while they create their own ATP and have their own DNA, mitochondria are often controlled by fusion and fission in the cell (Bereiter-Hahm & Voth 1994). Data shows that fusion and fission are necessary for mitochondrial morphology and normal biochemical function in a cell (Chen *et al.* 2003). Fragmentation occurs under conditions of unbalanced fission while unbalanced fusion gives rise to elongation (Chan 2006). Mitochondria are able to communicate and share with one another through fusion. An individual mitochondrion can be redefined by addition or removal of mitochondrial material through either fusion or fission, demonstrating that these organelles are semi-autonomous (Chan 2006).

Fusion, in the mitochondria, is the occurrence in which two coalesce to form one. The mitofusions Mfn1 and Mfn2, GTPases localized in the outer mitochondrial membrane, are essential for the fusion process to occur (Chen *et al.* 2003). Research has demonstrated that cells without Mfn1 or Mfn2 have dramatically decreased levels of mitochondrial fusion, while cells without both Mfn1 and Mfn2 result in no fusion, loss of tubules, and poor function in the mitochondria (Chan 2006). Both mitofusins appear to mediate the tethering of the mitochondria to one another during the fusion process although this mechanism is not known for sure (Figure 2) (Chan 2006). Fusion occurs when at least one tip or side of two mitochondria come into contact with one another (Bereiter-Hahm & Voth 1994). Research using electron microscopy has determined that once contact has been made there is an increase in high electron density relative to the areas that have come in contact (Bereiter-Hahm & Voth 1994). These areas are specialized zones for the fusion event. Mitofusions, on adjacent mitochondria, form *trans* complexes which assist in the mixing of mitochondrial matrix components. (Chan 2006). Crista junctions,

narrow tubules that connect cristae to the inner membrane, are considered to be involved in some way in the fusion of that inner membrane (Chen *et al.* 2003). It is known that a loss of the OPA1 (optic atrophy 1) protein leads to great deficiencies in cristae structure; therefore, this protein is thought to be critical in the fusion of the inner membrane (Chan 2006).

Fission, in mitochondria, is the process in which a single mitochondrion divides into two. It has been noted as similar to that of fission in bacteria and prokaryotes. The protein Drp 1 (dynamin protein 1), essential for mitochondrial fission, is found in the cytosol with a subpool localized to particular locations on mitochondrial tubules (Figure 3) (Chan 2006). Inhibition of Drp1, through either RNAi (RNA interference) or mutation, results in a mitochondrial tubules increased in length and entanglement (Chan 2006). Fis1 is also essential for fission, as knockouts of this protein had a similar effect on the mitochondria as demonstrated through inhibition of Drp1 (Chan 2006). Fis1 is contained to the outer membrane of the mitochondria with majority of the protein exposed to the cytosol and a single C-terminal transmembrane domain (Chan 2006). Fis1 attracts another protein, Dnm1 (Dynamin-1), which forms a complex with Drp1. The complex wraps around the mitochondria, in a molecular mechanism not well understood, and constricts the microtubules to mediate fission (Figure 4) (Chan 2006).

### **Mitochondrial DNA (MtDNA)**

Mitochondria contain their own small, circular genome. A group of scientists working under Frederick Sanger at Cambridge University published the Cambridge Reference Sequence (CRS) in 1981 (Anderson *et al.* 1981). It was at this time that the number of base pairs and functional genes were established. However, when the procedure was repeated by others, some discrepancies were discovered in the order of nucleotides in the sequence. The CRS was revised in 1999 to account for these errors and was renamed rCRS (Andrews *et al.* 1999). The mitochondrial genome (mtgenome) is made up of approximately 17,000 base pairs (Butler 2009). One can find hundreds to thousands of mtDNA

molecules within a single cell (Kavlick *et al.* 2011). MtDNA exists in human cells in varying quantity based on the amount of mitochondria in the cell. Most of the mtgenome codes for 13 proteins involved in the oxidative phosphorylation of cellular respiration which takes place within the inner mitochondria (Figure 5) (Falkenberg & Larsson 2009). These proteins are the subunits of cytochrome *c* oxidase, cytochrome *b*, and ATPase (Anderson *et al.* 1981). There is an 1122 base pair “noncoding region” which is the area of the genome that does not code for any genes that lead to protein production (Butler 2009). The non-coding region contains two hypervariable regions: hypervariable region 1 and hypervariable region 2 which are collectively known as the D-loop, control region, or hypervariable region. This hypervariable region has been reported to mutate at a 5-10 times higher rate than the marked areas of the nuclear genome (Butler 2009). It is this trait of demonstrated population specific variation that has allowed mtDNA analysis to be used for forensic analysis.

### **Forensic Analysis of MtDNA Variation**

While a mitochondrion typically contains only 0.25% of a cell’s total DNA, hundreds of mitochondria inhabit the cytosol of cells allowing mtDNA to be the most plentiful genetic material in forensic samples (Butler 2009). Current literature suggests that mtDNA is most useful during forensic investigations involving hair and calcified tissues in ancient samples, mass disasters and missing persons when DNA specimens are often environmentally compromised or highly degraded (Kavlick *et al.* 2011). The mtgenome’s size, shape, and copy number yield it more resistant to degradation than nuclear DNA which exists in a single copy in the nucleated cells (Kohnemann, Sibbing, Pfeiffer & Hohoff 2008). The odds of mtDNA forensic markers surviving cellular damage are greater than that of nuclear genome since there are hundreds to thousands of mtgenomes in each nucleated cell. MtDNA is also beneficial in cases in which the obtained extracted DNA sample is very small (Kavlick *et al.* 2011).

### **MtDNA Inheritance**

The numerous advantages of mtDNA are often ignored as its limited power of discrimination has been made more evident. Mitochondrial DNA does not undergo chromosomal recombination, Mendelian inheritance, or replication repair barring mutation (Butler 2009). A presumed shared “matrilineal ancestor” living in Africa approximately A.D 200,000 years ago is the source responsible for “all mtDNA types in the human gene pool” (van Oven & Kayser 2009). Accumulating mutations over time have brought about mtDNA sequence variation among maternal lineages (van Oven & Kayser 2009). MtDNA is passed through the generations independent of male influence as the fertilizing sperm only contributes cellular components directly to the nucleus (Figure 6) (Schwartz & Vissing 2002). The mitochondria of sperm usually disappear by “selective destruction, inactivation, or simple dilution” by that of the egg’s mitochondria (Schwartz & Vissing 2002). However, there have been a few unusual cases of paternally inherited mtDNA (mostly found in mice) that have been identified through PCR (Polymerase Chain Reaction) amplification and gel electrophoresis (Schwartz & Vissing 2002). Therefore, maternal offspring will inherit an identical mtgenome in most cases, excluding the possibility of mutation (Figure 7). For reasons previously discussed, the mitochondrion is not unique to an individual, which lowers its power of discrimination with regard to criminal cases. Another concern with mtDNA analysis in forensics is that hair and calcified tissue often contain melanin and humic acid, known PCR inhibitors (Kavlick *et al.* 2009). These foreign components, at certain levels, will hinder PCR amplification results.

### **MtDNA Haplogroups**

The control region of the mtgenome is enriched in sequence variation due to a higher overall mutation rate which allows researchers to create a mtDNA profile (Kohnemann, Sibbing, Pfeiffer & Hohoff 2008). The result of a mtDNA profile, is a mtDNA haplotype. A haplotype is regarded as alleles or traits that are inherited together. The genetic information lineage markers are known as haplotypes instead of genotypes as there is only a single allele per individual in mtDNA. A haplogroup (Hg) is a group of



haplotypes sharing common single nucleotide polymorphism (SNPs) mutations of an ancestor (Ennafaa & Vincente 2009). Most haplogroups are assigned based on SNPs and sequence variation found in the control region (Kohnemann, Sibbing, Pfeiffer & Hohoff 2008). Various positions (146, 150, 152, 195, 16189, 16311, 16362, 16519) appear to mutate at a rate faster than the rest of the mtgenome (van Oven & Kayser 2009). It is understood that from that first maternal figure in Africa, mtDNA sequential accumulation of common mutations have resulted in haplogroups which can be illustrated by a phylogenetic tree (van Oven & Kayser 2009). The L Hgs are the oldest lineages which are considered African specific. The L Hg is comprised of subgroups (L0, L1, L2, L3, L4, L5) with L3 giving rise to group M, N, and R (van Oven & Kayser 2009). Lineages of Hgs C, D, E, G, Q, and Z are subgroups of M, Hgs A, I, S, W, X, and Y are lineages within N, and B, F, HV, H, J, K, P, T, U, and V are within the Hg R (van Oven & Kayser 2009).

### **MtDNA Databases**

mtDNA population databases serve as a mean to approximate the expected frequency of haplotypes observed when an individual's mtDNA sequence matches that of a particular sample (Butler 2009). Many around the world have spent a great amount of time and resources to compile mtDNA samples from thousands of maternally unrelated samples to create databases. It is important for those creating the database to collect many, diverse samples. The databases must obtain "high-quality" information in order to make a reliable estimate of the incidence of a potential random match (Butler 2009).

Many publications have expressed concerns with the quality and validity of mtDNA databases. The ability to accurately generate frequency estimates for random matches in a forensic setting is of the utmost significance to forensic analysts (Butler 2009). Phylogenetics is used to systematically compare the similarities and differences between many related sequences (Butler 2009). These analyses can play a role in verifying sequence quality. Various errors can occur in establishing mtDNA population

databases. These mistakes range from in vivo errors in the course of transcription to sample mix ups, contamination, and the use of different nomenclatures when submitting to databases (Butler 2009). Mitochondrial DNA forensic databases are drastically lacking in sample size as well as population diversity. Research concerning HV1 sequence analysis in U.S European Americans show that approximately 39 to 50% of European Caucasians belongs to the haplogroup H (Kohnemann, Sibbing, Pfeiffer & Hohoff 2008). Studies have determined that mtDNA analysis may have a limited use in forensics as so many individuals fall into this Hg. Therefore, a further analysis was conducted to subtype haplogroup H into “subclades” based on a selection of particular SNPs (single nucleotide polymorphisms) that have a more “restricted geographic distribution” (Kohnemann, Sibbing, Pfeiffer & Hohoff 2008). Research did not consider U.S African Americans, for this population was minimally represented in mtDNA database samples and general studies by researchers in academia. Studying the mtgenome in U.S African Americans could be useful due to the diversity of the Hg on the African continent. Mitochondrial DNA analysis may prove to have a higher power of discrimination for this population. If we can catalogue this diversity in the African American population, it is therefore possible that mtDNA analysis may be used to exonerate individuals previously accused of crimes.

The FBI’s mtDNA database contains 4839 mtDNA profiles from 14 different ethnic populations that were mainly focused on positions 16,024 to 16,365 in HV1 and 73 to 340 in HV2 (Butler 2009). Mitosearch, a tool that can be downloaded with the Microsoft Access format of the FBI’s mtDNA population database, was released in 2002 (Butler 2009). This tool reviews the population data for specific sequences that are entered with regards to disparities in the rCRS and tells the user the number of times that the indicated profile appears in each ethnic population group (Figure 8) (Butler 2009).

EMPOP:

The European DNA Profiling Group mitochondrial DNA population database project (EMPOP) was underway several years before that of the United States (Butler 2009). This database can be accessed by the public at <http://empop.org> . The database is steadily growing; as of December 2008, there were 4527 high-quality mtDNA data online, while as of December 2011, there are 16,121 haplotypes available (EMPOP 2011).

mtDNAManager:

mtDNAManager, an online mtDNA population database with a search engine, was created by a group of people from Yonsei University in Seoul Korea (Butler 2009). This program allows users to “analyze, query, and store human mtDNA control region sequences (mtDNAManager 2012). mtDNA manager can be assessed at <http://mtmanager.yonsei.ac.kr> . The site is free and open to any users without having to login to the site (mtDNAManager 2012). The program offered 7090 mtDNA sequences of the control region as of December 2008 (Butler 2009).

## **Research Aim**

Optimal enzymatic conditions must be defined in order to conduct research determining the resolving power of mtDNA. Standard PCR enzymes and primers do not account for the underrepresented African Americans. In this study, DNA was extracted and the samples were amplified under several different PCR conditions, once cleaned. The PCR polymerase enzymes, with appropriate conditions, used in this experiment were ABI™ , Kapa HiFi Hot Start, Phusion® High Fidelity, Platinum® *Taq*, and FastStart High Fidelity. The PCR products were run on a 1% agarose gel by electrophoresis to verify the success of each enzyme. A UV photo of the gel was taken with an indication of a “band” in each well showing the efficiency of the enzyme. Samples that produced PCR product under the specific enzyme conditions were then purified by Qiagen PCR clean up. The PCR product was sequenced, using Sanger sequencing techniques, to be used toward analysis of the mtgenome.

In this study we aim to optimize PCR conditions for mtDNA HV1 SNP amplification. Once determining the most efficient conditions, we will eventually be able to use this method (s) of PCR in discovering whether mtDNA analysis is beneficial in forensics with regards to U.S African Americans.

### **Methods and Materials:**

#### **DNA Extraction from Buccal Swab**

DNA was obtained using sterile, single use swabs by rubbing along the inside of the cheek of the mouth for 20 seconds. Two swabs were used for each sample as one swab was used for each cheek. The tip of each swab was discarded into a labeled 1.5 ml microcentrifuge tube. Five hundred  $\mu$ l of Human Extraction Buffer (10mM EDTA, 2% SDS pH=7.5, 10mM Tris pH=7.5, 50mM NaCl) and 15  $\mu$ l of Proteinase K (15ng/ $\mu$ l) were added to each tube. Each tube was vortexed in the Vortex- Genie 2 by Scientific Industries and centrifuged in the MicroV: Fisher Scientific microcentrifuge. In Barnstead/ Thermolyne Type 17600 Dri-Bath, the tubes were incubated at 56°C for 2 hours. After incubation, the swab was removed and placed into a SPIN-EASE basket. The basket was placed in the MicroV Fisher: Scientific microcentrifuge for 20 seconds at 9,000 rpm/g. The swab and spin basket were discarded after. To the tubes, 450  $\mu$ l of PCI (phenol/chloroform/isoamyl alcohol) was added. They were then inverted 5 times and a milky emulsion appeared. Using the same centrifuge, the tubes were spun at 9,000 rpm/g for 5 minutes. The supernatant was removed and placed into a new microcentrifuge tube. The new tubes were placed back in the microcentrifuge to repeat these last steps one more time.

#### **Microcon- 100 Purification and Concentration**

To each tube, 500  $\mu$ l of water –saturated-n-butanol was added. The tubes were inverted 5 times and until a milky emulsion appeared. The contents were centrifuged for 5 minutes at 9,000 rpm/g. The supernatant was discarded into a beaker labeled “Phenol”. The bottom layer was applied to a Microcon-100 unit filter. Using the Eppendorf Centrifuge 5415D, the tubes were centrifuged at 4.6 rpm for 20 minutes. Filtrate was discarded and 400  $\mu$ l of distilled water was added to the filter. The tubes were

placed back in the previous centrifuge, and the previous last steps were repeated. Directly to the filter, 50 µl of distilled water was added before inverting and setting the tubes inside a clean microcentrifuge tube. At 4.6 rpm in the same centrifuge, the tubes were spun for 2 minutes. The DNA can then be stored for use at 40°C.

### **PCR Conditions**

For each PCR reaction, a negative sample was also run with the mtDNA samples. A negative contains all of the ingredients of each PCR reaction with the exception of the mtDNA template. Distilled water was added in the mtDNA's place at the appropriate volume.

#### **HV1 ABI™ DNA Polymerase**

Each PCR tube was filled with 20 µl; the contents in each tube were: 2.5 µl of 10X PCR Buffer, 1.5 µl of 25 mM MgCl<sub>2</sub>, 2.5 µl of 2mM dNTPs, 0.5 µl of 5 µM HV1 forward primer, 0.5 µl of 5 µM HV1 reverse primer, 0.2 µl of 5 U/µl *Taq* polymerase (which must always stay on ice), and 12.3 µl of milli-Q water. To each tube, 5 µl of the corresponding diluted DNA was added. Each tube was vortexed in the Vortex-Genie 2 by scientific industries and spun down in the Eppendorf Centrifuge 5415D before being placed in either the DNA Engine Dyad™ Peltier Thermal Cycler or Bio Rad My Cycler™ Thermal Cycler.

The HV1 ABI™ PCR conditions are as follows:

- 1) 94°C for 2 minutes
- 2) 94°C for 45 seconds
- 3) 52°C for 30 seconds
- 4) 72°C for 1 minute
- 5) Repeat steps (2-4) 30 times
- 6) 72°C for 5 minutes
- 7) 10°C hold

#### **HV2 ABI™ DNA Polymerase**

Each PCR tube was filled with 20 µl; the contents in each tube were: 2.5 µl of 10X PCR Buffer, 1.5 µl of 25 mM MgCl<sub>2</sub>, 2.5 µl of 2mM dNTPs, 0.5 µl of 5 µM HV2 forward primer, 0.5 µl of 5 µM HV2 reverse

primer, 0.2 µl of 5 U/µl *Taq* polymerase (which must always stay on ice), and 12.3 µl of milli-Q water. To each tube, 5 µl of the corresponding diluted DNA was added. Each tube was vortexed in the Vortex-Genie 2 by scientific industries and spun down in the Eppendorf Centrifuge 5415D before being placed in either the DNA Engine Dyad™ Peltier Thermal Cycler or Bio Rad My Cycler™ Thermal Cycler.

The HV2 ABI™ PCR conditions are as follows:

- 1) 94°C for 2 minutes
- 2) 94°C for 45 seconds
- 3) 59°C for 30 seconds
- 4) 72°C for 1 minute
- 5) Repeat steps (2-4) 30 times
- 6) 72°C for 5 minutes
- 7) 10°C hold

Kapa HiFi Hot Start Polymerase

Each PCR tube was filled with 15 µl; the contents in each tube were: 5.0 µl of 5X Kapa HiFi Fidelity Buffer, 0.75 µl of 10mM Kapa dNTP mix, 0.75 µl of 10 µM forward primer, 0.75 µl of 10 µM reverse primer, 0.5 µl of Kapa HiFi Hot Start Polymerase, and make up to 15 µl of PCR- grade water. To each tube, 10 µl of the corresponding diluted DNA was added. Each tube was vortexed in the Vortex-Genie 2 by scientific industries and spun down in the Eppendorf Centrifuge 5415D before being placed in either the DNA Engine Dyad™ Peltier Thermal Cycler or Bio Rad My Cycler™ Thermal Cycler.

The Kapa HiFi Hot Start PCR conditions are as follows:

- 1) 95°C x 5 minutes – hot start
- 2) 98°C for 20 seconds
- 3) 65°C for 15 seconds
- 4) 72°C for 15 seconds
- 5) 72°C for 1 minute
- 6) Repeat (2-5) 25 times
- 7) 10°C hold

Phusion High Fidelity DNA Polymerase

Each PCR tube was filled with 20  $\mu$ l; the contents in each tube were: 4  $\mu$ l of 5X Phusion High Fidelity or GC Buffer, 0.4  $\mu$ l of 10 mM dNTPs, 1  $\mu$ l of 10  $\mu$ M forward primer, 1  $\mu$ l of 10  $\mu$ M reverse primer, 0.2  $\mu$ l of Phusion DNA polymerase, and make up to 20  $\mu$ l of nuclease-free water. To each tube, 5  $\mu$ l of the corresponding diluted DNA was added. Each tube was vortexed in the Vortex-Genie 2 by scientific industries and spun down in the Eppendorf Centrifuge 5415D before being placed in either the DNA Engine Dyad™ Peltier Thermal Cycler or Bio Rad My Cycler™ Thermal Cycler.

The Phusion High Fidelity PCR conditions are as follows:

- 1) 98°C for 30 seconds
- 2) 98°C for 10 seconds
- 3) 45-72°C for 30 seconds
- 4) 72°C for 30 seconds
- 5) Repeat steps (2-4) 25-35 times
- 6) 72°C 10 minutes
- 7) 10°C hold

Platinum *Taq* DNA Polymerase- High Fidelity Storage Buffer

Each PCR tube was filled with 24  $\mu$ l; the contents in each tube were: 2.5  $\mu$ l of 10X High Fidelity PCR Buffer, 1  $\mu$ l of 50 mM  $\text{MgSO}_4$ , 0.5  $\mu$ l of 10 mM dNTP mixture, 1  $\mu$ l of 10  $\mu$ M primer mixture, 0.1  $\mu$ l of Platinum *Taq* High Fidelity polymerase, and made up to 24  $\mu$ l of distilled water. To each tube, 1  $\mu$ l of the corresponding diluted DNA was added. Each tube was vortexed in the Vortex-Genie 2 by scientific industries and spun down in the Eppendorf Centrifuge 5415D before being placed in either the DNA Engine Dyad™ Peltier Thermal Cycler or Bio Rad My Cycler™ Thermal Cycler.

The Platinum *Taq* DNA Polymerase PCR conditions are as follows:

- 1) 94°C for 2 minutes
- 2) 94°C for 30 seconds
- 3) 55°C for 30 seconds
- 4) 68°C for 1 minute
- 5) Repeat (2-4) for 25-35 cycles
- 6) 10°C hold

### FastStart High Fidelity Enzyme

Each PCR tube was filled with 24  $\mu$ l; the contents in each tube were: 2.5  $\mu$ l of 10X Fast Start High Fidelity Reaction Buffer, 0.5  $\mu$ l of PCR Grade nucleotide mix, 2.5  $\mu$ l of forward primer, 2.5  $\mu$ l of reverse primer, 0.25  $\mu$ l Fast Start High Fidelity Enzyme Blend, and made up to 24  $\mu$ l of distilled water. To each tube, 1  $\mu$ l of the corresponding diluted DNA was added. Each tube was vortexed in the Vortex-Genie 2 by scientific industries and spun down in the Eppendorf Centrifuge 5415D before being placed in either the DNA Engine Dyad™ Peltier Thermal Cyclor or Bio Rad My Cyclor™ Thermal Cyclor.

The FastStart High Fidelity Enzyme PCR conditions are as follows:

- 1) 94°C for 2 minutes
- 2) 94°C for 30 seconds
- 3) 55-68°C for 30 seconds
- 4) 68°C for 3-5 minutes
- 5) Repeat (2-4) for 25-40 times
- 6) 68°C for 7 minutes
- 7) 10°C hold

### Verification of PCR products/ 1% Agarose Gel

In a microwaveable flask, 1 g of agarose is poured and then the final volume is brought to 100 ml using 1X TBE buffer. The flask is heated in the microwave until the agarose is dissolved and the solution appears clear. After allowing the flask to cool to 50°C, 1  $\mu$ l of ethidium bromide (for every 100 ml of TBE) is added. The flask was swirled gently before casting the gel and set for 20 minutes. While the gel was being cast, 2  $\mu$ l of loading dye was added to 5  $\mu$ l of PCR product and centrifuged in MicroV Fisher: Scientific microcentrifuge. Once the gel has been cast, all 7  $\mu$ l of PCR product and loading dye was added to the wells in the gel. Also, a 2  $\mu$ l kb ladder was added. The gel was run at 140 V for 60 minutes. The PCR products were visualized under UV light in the Bio Rad UV camera.

### Purification of amplification products (Qiagen PCR clean up)



To the PCR product, 100 µl of PB buffer was added. The entire mixture was applied to the QIAquick column and centrifuged for 60 seconds at 13,200 rpm/g in the Eppendorf Centrifuge 5415D. Flow-through was discarded. To the QIAquick column, 750 µl of Buffer PE was added. The tubes were centrifuged at 60 seconds at 13,200 rpm/g in the Eppendorf Centrifuge 5415D. The flow-through was once again discarded and the column was placed back in the tube to be centrifuged with the same machine over the same conditions again. The QIAquick column was then placed in a clean 1.5 ml microcentrifuge tube. To the center of the filter, 30 µl of milli-Q water was added and then let stand for 2 minutes. To collect the purified DNA, the tubes were centrifuged for 1 minute at 13,200 rpm/g in the Eppendorf Centrifuge 5415D. The column was discarded; the DNA in the microcentrifuge tube was then stored for further experimentation.

#### **Sanger Sequencing PCR set up (ABI™)**

A forward-primer master mix and reverse-primer master mix were created according to the following per tube: 0.5 µl of Big Dye Terminator, 1.75 µl of 5X Big Dye Buffer, 1.0 µl of 3.2 pmol M13 forward or reverse primer, and 5.75 µl of distilled water. In each PCR tube, 9 µl of the appropriate master mix and 1 µl of purified PCR product were mixed. Each DNA sample had a tube with a forward and reverse primer mixture. The PCR tubes were centrifuged for 1 minute by the MicroV Fisher: Scientific microcentrifuge and then loaded into either the DNA Engine Dyad™ Peltier Thermal Cycler or Bio Rad My Cycler™ Thermal Cycler for sequencing.

ABI™ Sanger Sequencing PCR conditions are as follows:

- 1) 96°C for 1 minute
- 2) 96°C for 10 seconds
- 3) 50°C for 5 seconds
- 4) 60°C for 4 minutes
- 5) Repeat steps (2-4) 30 times
- 6) 10°C hold

### **Precipitation of Sequence PCR Products (Qiagen DyeEX kit)**

The Dye Ex spin columns that come with the kit are vortexed in the Vortex- Genie 2 by Scientific Industries. The caps were loosened a quarter of a turn and the bottom was snapped off before being placed in the 2 ml collection tube provided by the kit. For 3 minutes, the tubes with the columns were centrifuged at 3,000 rpm for the Eppendorf Centrifuge 5415C. The Dye Ex spin columns were then placed in a new 1.5 ml microcentrifuge tube. To the gel bed of the column, 10 µl of the sequencing reaction product was added. At 3,000 rpm on the Eppendorf Centrifuge 5415D, the tubes were spun down for 3 minutes. Once the spin columns had been discarded, the open tubes were put on the hot plate and covered with aluminum foil for about 30 minutes until all the liquid had evaporated. After every tube had been completely dry, the pellet was resuspended in 20 µl of Hi-Di formamide. The tubes were vortexed by the Vortex- Genie 2 by Scientific Industries for 20 seconds. The contents of the tubes was then transfer to the sequencer plate. This plate was then loaded into the ABI 3130 sequencer to be sequenced.

Four samples were extracted and purified according to the procedure previously described in this section. The samples were named as “007”, “1087”, “721”, and “1987”. The following primers were used to generate mtDNA amplicons:

Suhl, (2008): Forward: 5'-TGT AAA ACG ACG GCC AGT CAA ATC AGA GAA AAA GTC TT-3'

Reverse: 5'-CAG GAA ACA GCT ATG ACC TTG TGC GGG ATA TTG ATT TC-3'

Levin *et al.*, (2003) Forward: 5' -CAC CAT TAG CAC CCA AAG CT- 3'

Reverse: 5'-GAG GAT GGT GGT CAA GGG AC- 3'

These primers were evaluated under ABI™ PCR conditions to determine which were more optimal to use in the further experimentation. This procedure was performed twice to confirm the efficiency of each

primer set. In each procedure, the samples were tested twice. Results (Figure 9 and Figure 10) indicated that both primers yielded product. It was decided that the primers described by Levin *et al.* (2003) were to be used in the testing of the different PCR conditions.

### **Results:**

To test the efficiency of various polymerases, four samples were subjected to PCR analysis, each with the varying PCR conditions. The following polymerases were analyzed: Kapa HiFi Hot Start, Fast Start High Fidelity, Phusion® High Fidelity, and Platinum® *Taq*. Appropriately sized mtDNA fragments, corresponding to the HV1 region, were obtained for each sample using the Platinum® and Fast Start polymerases. Kapa HiFi Hot Start yielded samples 1087 and 007 (Figure 11). Testing samples with Platinum® (n=8), Phusion® (n=4), and Fast Start (n=4), produced 12 bands (Figure 12). All of the samples tested worked with Platinum® and Fast Start; yet, with Phusion® none of the samples generated bands under the UV photo under the gel electrophoresis. The procedure was repeated exactly and yielded identical results (figure not shown).

The samples that gave PCR amplification product were then purified using the materials and instructions in the Qiagen kit. They were further amplified via Sanger ABI™ PCR conditions and precipitated using the Qiagen Dye-Ex kit. The resultant contents were placed in the ABI 3130 sequencer, where the nucleotide sequences of each mtDNA sample was generated by the computer. The obtained sequential information proved beneficial with regards to determining sample haplogroups and SNPs. This information for the samples used in this research can be found on Figure 13 in the Appendix.

### **Discussion:**

The objective of this research is to combine mtDNA HV1 region amplification with standard Sanger sequencing to evaluate the power of discrimination of mtDNA analysis. Finding the most efficient

polymerases and PCR conditions for such amplification allows further investigation into the mtgenome. The mtDNA primers described by Levin *et al.* (2003) were chosen for these procedures over the primers defined by Suhl (2008) because they were more efficient at yielding the HV1 fragments (2003). The two primers amplify two different length regions: from 16024-16365 bp with Suhl (2008) primers and from 16024- 16569 bp with those by Levin *et al.* (2003). It was important to consider both the primer sequence and the primer length when optimizing PCR conditions to amplify the samples. Because there is a higher genetic variation in modern African populations, it was hypothesized that a U.S African American population would demonstrate similar variability. In such cases, primers that were specific to the site of interest and generated fewer instances of non-specific binding were ideal. While the initial experiments of this project proved that both primer pairs were efficient in generating mtDNA HV1 PCR amplicons in all samples, the Levin *et al.* (2003) primers were selected to be used with the understanding that, in the future, this research would eventually involve low copy number mtDNA samples. Literature suggested that using these primers to amplify low starting templates of DNA would be beneficial in comparison to actual forensic samples (Levin *et al.* 2003). The Levin *et al.* (2003) primers are used in FBI and state crime labs around the country. As these primers would be used in the future, when analyzing mtDNA in U.S African Americans, it was logical to employ them in conducting this research.

Two of the samples used in this experiment were self-identified U.S African American's (samples 1087 and 1987) while the other two samples self-identified as U.S European American. Sample demographics can be found in Figure 14 in the Appendix. It was important to use an ethnically diverse sample set in this research, as it is a future goal to be able to find amplification conditions that will be optimal for both. After testing the conditions of the four polymerases, it was determined that the samples amplified with Platinum® *Taq* Polymerase High Fidelity and Fast Start High Fidelity enzyme were the most successful. No amplification product was visible using gel electrophoresis for samples amplified with

Phusion® High Fidelity DNA polymerase. The Kapa HiFi Fast Start polymerase only produced two bands throughout the three experiments in which those enzymatic conditions were used.

Platinum® *Taq* Polymerase High Fidelity and Fast Start High Fidelity enzyme, according to the results of these experiments, yielded PCR products and were deemed to be most optimal when amplifying mtDNA HV1 fragments with the dataset used. Several similarities between the two polymerases provide a suggestion as to their efficiency. Both of these polymerases require only 1µl of purified mtDNA in a total reaction volume of 24µl. This sample amount is significantly less than that of ABI™(5µl), Phusion® High Fidelity Enzyme Polymerase (5µl), and Kapa HiFi Hot Start Enzyme (10µl). Comparatively, Platinum® *Taq* Polymerase High Fidelity and Fast Start High Fidelity cycle conditions are almost indistinguishable. The denaturation (94°C), annealing (55°C), and extension (68°C) temperatures match between the two polymerases. The only differences lie in the length of time at the extension temperature; Fast Start polymerase compels the samples to maintain this temperature for approximately three times as long as Platinum® *Taq* polymerase. Platinum® *Taq* and Fast Start polymerase conditions both also need 2.5µl of their respective reaction buffers which are at the same concentration (10X). These similarities may reveal why these two were most efficient in mtDNA PCR amplification. The Fast Start products contained multi-length fragments, as seen by the range of bands generated at each well (Figure 12). This may be fixed by shortening the length of the extension stage of the PCR reaction. Platinum® *Taq* worked well under the same PCR condition with a significantly shorter extension time, indicating the possibility that the Fast Start polymerase would work as well.

Phusion® High Fidelity DNA polymerase did not work on any of the samples under any of the procedures it was involved in. Kapa HiFi Hot Start produced two bands during the course of the experiments, only one band (sample 1087) being significant. The Phusion® polymerase protocol asked for 5X High Fidelity (or GC) reaction buffer, 5µl of sample mtDNA, and 3% DMSO in the reactants for PCR amplification;

while the Kapa HiFi Hot Start protocol similarly asked for 5X HiFi Fidelity reaction buffer, 10µl of sample mtDNA, and DMSO in the reactants for PCR amplification. The concentration of the reaction buffer for both enzymes may have been too dilute for the samples (Platinum® and Fast Start had 10X). It is also possible that the optional GC reaction buffer could have been more effective under the circumstances. Requiring such a substantial volume of mtDNA implies that there are fewer amounts of the reactants in each 25µl reaction; there may not have been enough reactants to amplify the large amount of mtDNA. Yet, if these two polymerases need so much mtDNA, they may not be ideal for working with forensic samples. Forensic samples are typically found in significantly small amounts with very low copy numbers. The Kapa polymerase, for example, needs 10 µl of sample mtDNA for each PCR reaction. This volume is ten times greater than that needed for Platinum® and Fast Start procedures, emphasizing the ineffectiveness of the Phusion® and Kapa HiFi enzymes. Dimethyl sulfoxide (DMSO), although called for in the protocol, was not added during Phusion® High Fidelity reaction conditions until the third experiment the polymerase was being tested in. The addition had no effect upon the results for the enzyme. DMSO was not used in any of the experiments with Kapa HiFi Hot Start.

The PCR cycle conditions for Phusion® High Fidelity DNA polymerase and Kapa HiFi Hot Start polymerase differed greatly from that of the successful polymerases. Kapa requires a 5 minute hot start at 95°C before a denaturing period at 98°C. Phusion® also has a 98°C denaturation temperature. The two polymerases call for a 72°C extension temperature. Both the denaturation and extension temperatures are noticeably higher than those for Platinum® and Fast Start which possibly contribute to their lack of performance.

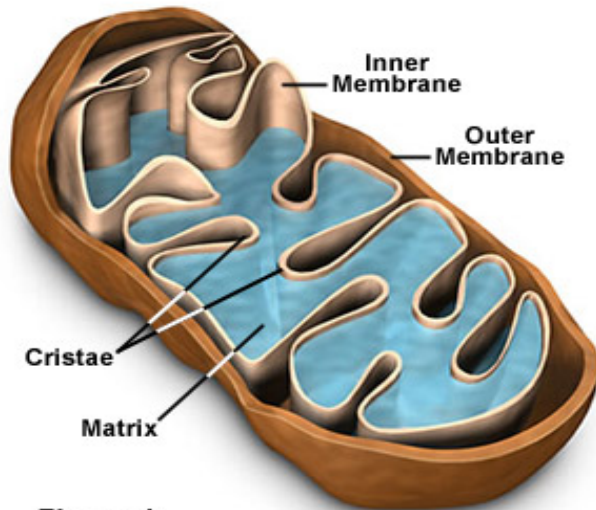
The Phusion® protocol recommended to assemble all of the components on ice and to transfer them quickly to the thermocycler that had been previously preheated to 98°C. This indicates that the polymerase is extremely sensitive to change in temperature which may have contributed to its

ineffectiveness during this particular study. It was observed throughout experimentation that the Phusion® High Fidelity polymerase is a “sudsy” enzyme, meaning that upon adding the enzyme to other contents, it forms bubbles similar to those seen in hand soap. The “suds” do not allow for very accurate pipetting or mixing of reaction contents. The polymerase should be added to the tubes separately from the master mix if this experiment were to be repeated.

The Kapa HiFi Hot Start enzyme cannot be determined unproductive for mtDNA PCR amplification based on this research due to the fact that after two tests, the lab ran out of the material for the Kapa HiFi kit and did not receive enough funding to buy more. More supplies would have to be obtained to officially exclude the Kapa polymerase for being optimal. The protocol for Kapa HiFi polymerase called for its own dNTP mix which may have affected its amplification. This dNTP mix, however, was 10mM like the ones used with the other polymerases. The Fast Start High Fidelity kit also had its own special dNTP mix and this did not hinder this enzyme from producing amplicons.

## Appendix:

### Mitochondria Structural Features



**Figure 1**

Figure 1. Internal Structural Features of the Mitochondria (Davidson 2010)

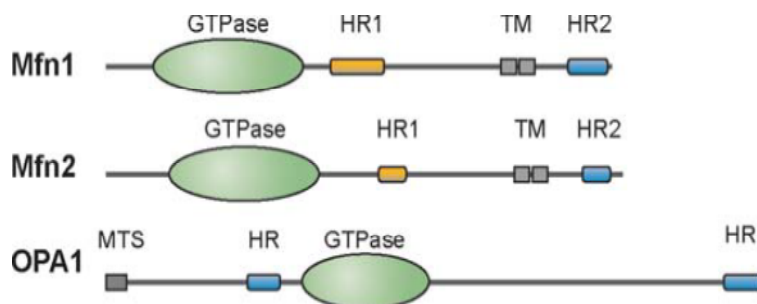


Figure 2. Mitochondrial Fusion Machinery: Structure of Fusion Proteins (Chan 2006)

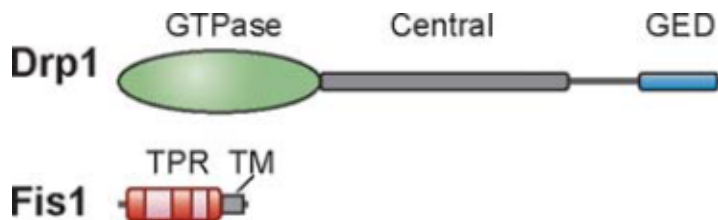


Figure 3. Mitochondrial Fission Machinery: Structure of Fission Proteins (Chan 2006)



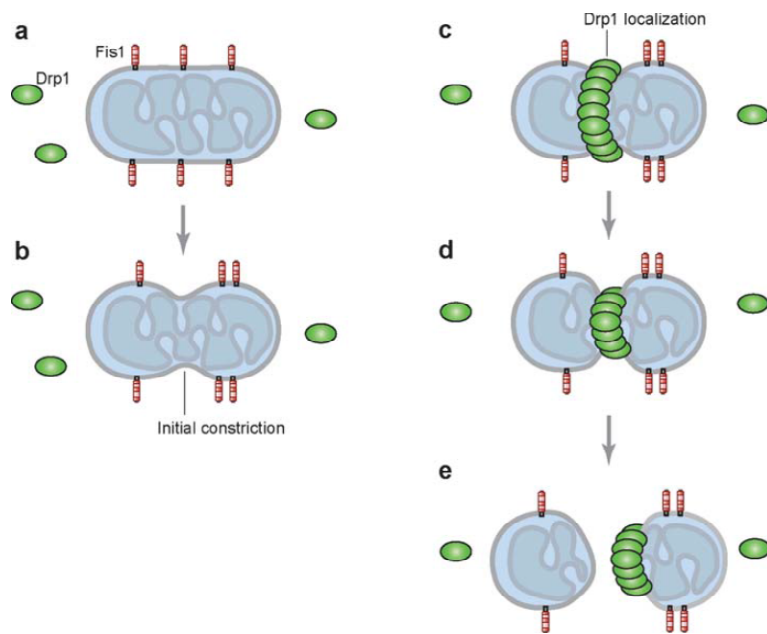


Figure 4. Mitochondrial Fission Mechanism with Fis1 and Drp1 (Chan 2006)

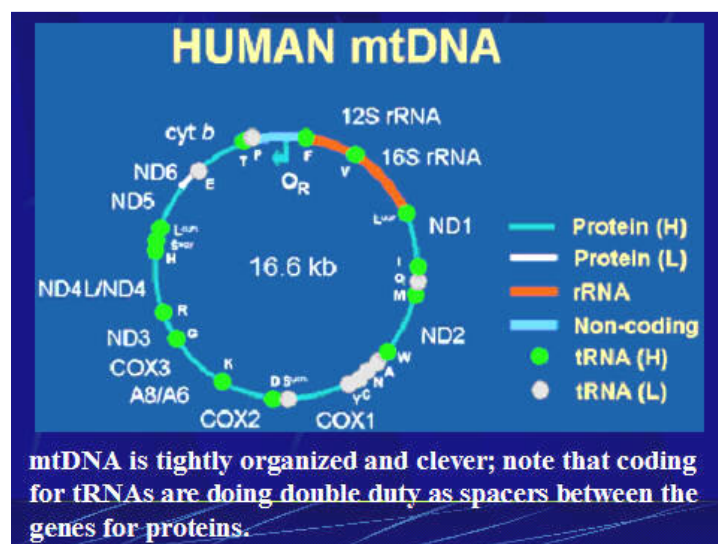


Figure 5. Genes, rRNA, and tRNA coded in Human Mitochondrial DNA (Childs 2003)

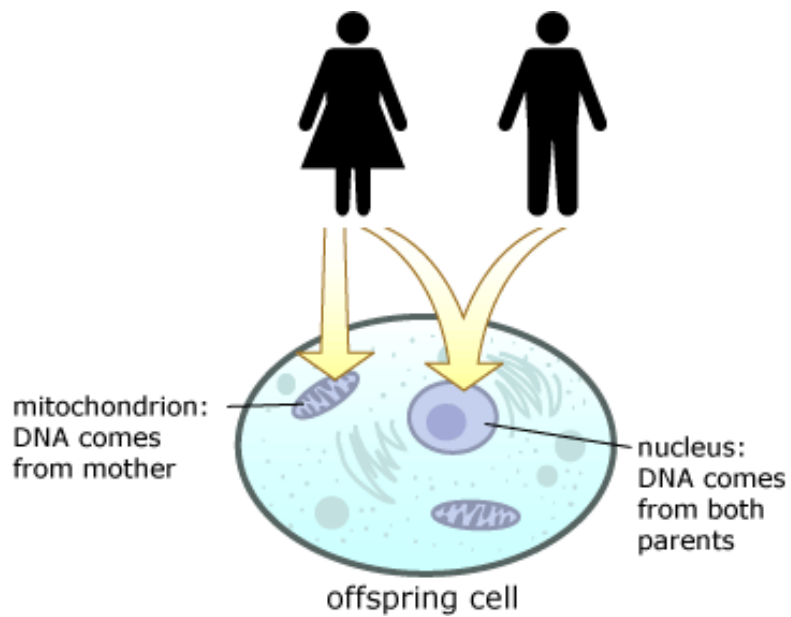


Figure 6. MtDNA Inheritance (University of California Museum of Paleontology's Understanding Evolution 2007)

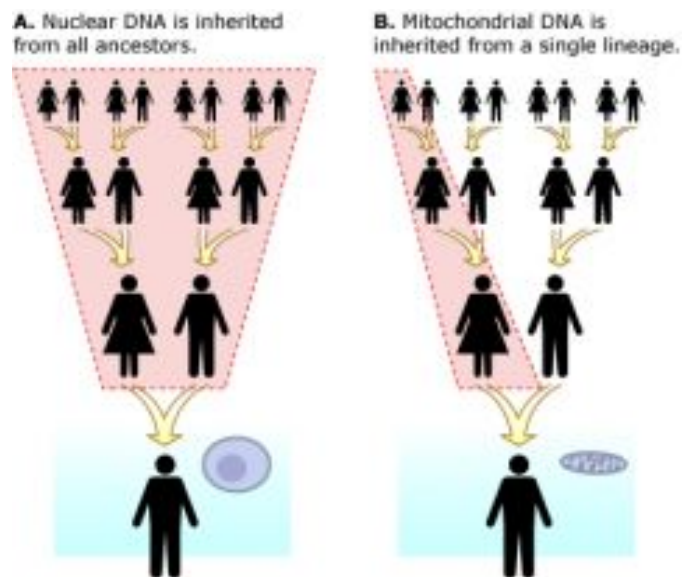


Figure 7. MtDNA Inheritance Through Ancestors (University of California Museum of Paleontology's Understanding Evolution 2007)

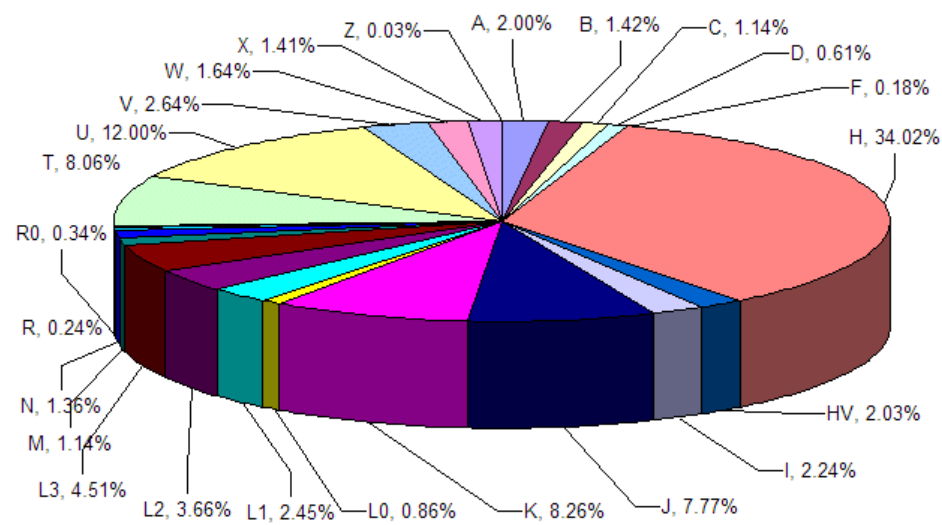


Figure 8. Haplogroup Approximate Frequencies in Mitosearch Database (Mitosearch 2011)

Sample bands found in the following photos are at ~ 500 bp according to the 1 kb ladder (farthest right):

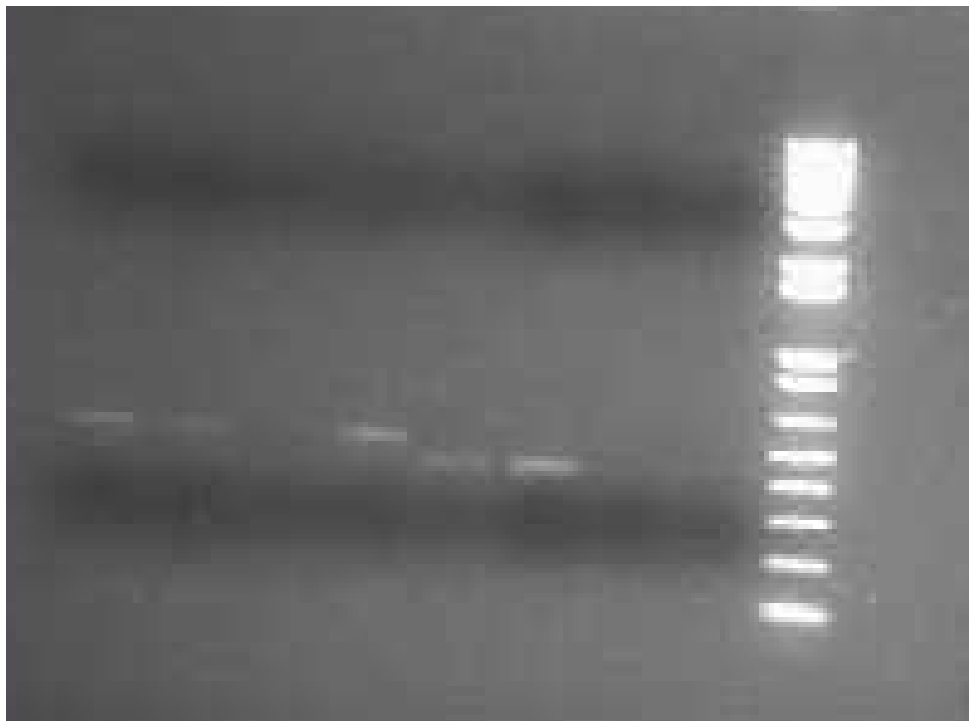


Figure 9. Procedure 1: Bands of Samples with Suhl (2008) and Levin *et al.* (2003) Primers (11/10/11)



Figure 10. Procedure 2: Bands of Samples with Suhl (2008) and Levin *et al.* (2003) Primers (11/11/11)

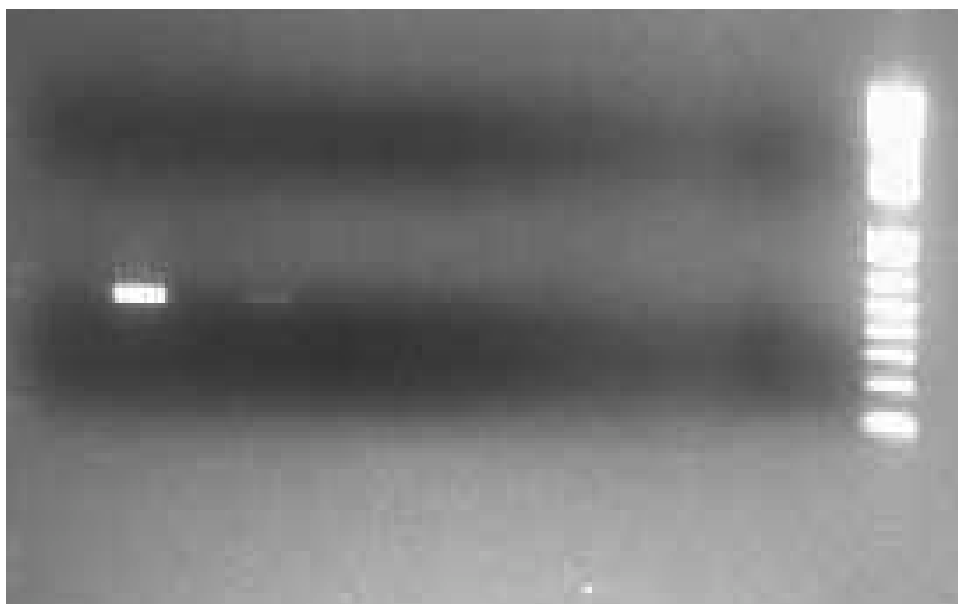


Figure 11. PCR Product from Samples "1087" and "007" under Kapa HiFi Hot Start Polymerase: No Product from Phusion® Samples (11/17/11)

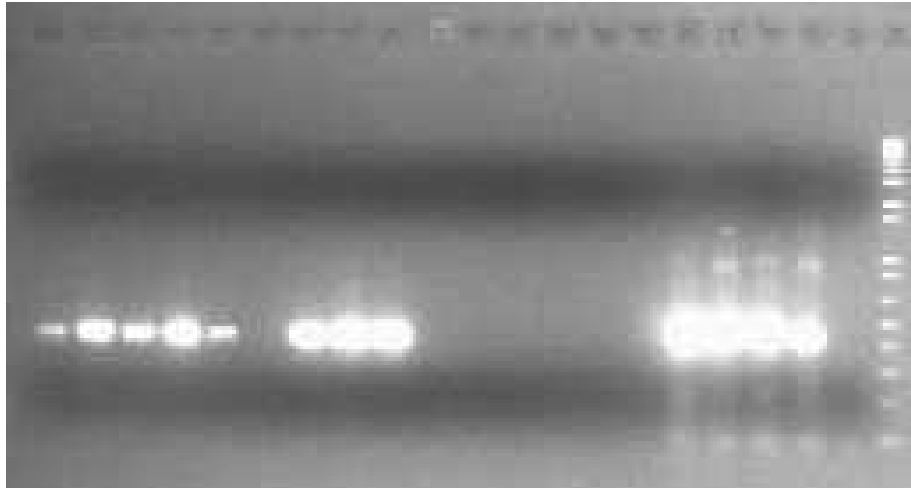


Figure 12. PCR Product from Samples using Taq Platinum® (first 8 bands) and Fast Start High Fidelity (last four bands): No Product from Phusion® Samples (12/5/11)

Sample Name	Haplogroup	SNPs
1987	L2b	d16031
		16114A
		16129A
		16213A
		16223T
		16278T
		16325C
		i16355C
		16362C
1087	L3e3	16223T
		16249C
		16265T
007	H	none

Figure 13. Haplogroups and SNPs determined from sequences obtained from experiment samples

Sample	"1087"	"721"	"1987"	"007"
Gender	F	F	F	F
Ethnic Group	African American	European American	African American	European American
Age	29	23	19	31
Place of Birth	California	Greece	Connecticut	Connecticut
Mother's Place of Birth	Florida	Connecticut	Jamaica	Connecticut
Maternal Grandmother's Place of Birth	Florida	Connecticut	Jamaica	Northern Italy
Maternal Grandfather's Place of Birth	Florida	Connecticut	Jamaica	Northern Italy
Father's Place of Birth	Alabama	Connecticut	Jamaica	Louisiana
Paternal Grandmother's Place of Birth	Alabama	Connecticut	Jamaica	unknown
Paternal Grandfather's Place of Birth	unknown	Connecticut	Jamaica	unknown

Figure 14. Demographics of the Individuals of the four mtDNA Samples

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